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DETERMINATION OF POLYPLOIDY FROM HERBARIUM SPECIMENS

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In the evolutionary history of the flowering plants there are several biological phenomena known to be of major consequence. Foremost among these, and perhaps the best understood, is polyploidy. A brief survey of any of the recent compilations of chromosome numbers of the Angiosperms (Löve and Löve, 1948, Delay, 1951; Darlington and Wylie, 1955) will suffice to show the frequency of this phenomenon. There are literally hundreds of examples of so called intraspecific polyploids reported, not to mention the even more frequent condition of interspecific polyploidy.

The importance of polyploidy in the critical evolutionary fields of taxonomy, geobotany, hybridization, mode of reproduction, etc. has been shown and discussed by many workers (Löve, 1951; Löve and Löve, 1949; Gustafsson, 1947; 1948; Muntzing, 1936; Stebbins, 1940; 1950; Darlington, 1956, etc.). Not only is the importance of polyploidy well known but much is understood concerning its biological mechanisms of operation.

Because of the obvious significance of polyploidy in both continuous and discontinuous variation of plants, it is a factor that cannot easily be dismissed in any detailed study involving the relationships of species or their modes of origin.

Determination of chromosome numbers is, however, a time consuming operation, and becomes virtually impossible for monographers who deal principally with non-living herbarium materials. Some effort has been made to overcome this handicap and recently Khoshoo (1955) has been able to study chromosomes

from herbarium material in *Impatiens*. However the techniques are rather laborious, the results far from the best, and the extent to which the technique is applicable is not yet known.

Numerous studies have been conducted that attempt to correlate morphological conditions with degree of ploidy (see Stebbins, 1950 for review), and some workers believe that there is almost always some correlation (Löve, 1951). Although the general conclusion from these studies is that there are no universal criteria, nevertheless there are certain characters that have rather general application. Foremost among these is cell size.

From herbarium material there are usually easily available two types of cells (pollen grains and guard cells of the stomata). Since there is often some overlapping between cell size and degree of ploidy, it is desirable, and sometimes essential, to study the cell size of both before drawing conclusions.

In general, pollen grains are easily studied, but the conventional method for studying guard cells requires a pretreatment of the leaf, followed by scraping or stripping (deWet, 1954). In any event some portion of the specimen is mutilated or destroyed, a condition that is very undesirable especially with valuable specimens such as types.

In this report an impression method is described for the study of the guard cells of the stomata that is quick, reliable, and causes no damage to the specimen. This method is based on the principles of impression long used in paleontology, and with the use of some of the modern plastics gives very desirable results. Somewhat similar methods have been used by plant pathologists (Long & Clements, 1934; Husain, 1956) to detect the open or closed condition of the stomata.

The procedure is simply to mix cellulose nitrate[1] in acetone until a viscous solution is obtained. When the constancy of the solution is such that it spreads smoothly with a camels hair brush, paint the surface of the leaf to be studied with the solution. This will work better if the leaf surface has been previously cleaned with acetone. The solution is allowed to dry thoroughly and is then peeled off with tweezers. This plastic strip is then

[1] Jacobo Ortega Castro of this department, in a study involving the relationship between the opening and closing of the stomata in wheat and leaf rust infection, has used cellulose acetate and collodion with equal success. His work shows that at different temperatures different plastics are preferable.

floated in a drop of water on a slide, covered with a cover slip, and is ready for study under the microscope.

Cell impressions prepared by this method are usually distinct (figs. 1–8) and measurements can be made with considerable confidence.

Results

In the present study several grass species complexes of the tribe *Andropogoneae* were analyzed. A rather detailed study was made of the *Dichanthium annulatum* complex, which included diploids ($2n = 20$), tetraploids ($2n = 40$), and hexaploids ($2n = 60$). Also studied, but in less detail, were tetraploids, pentaploids, and hexaploids of the *Bothriochloa ischaemum* complex, and tetraploids and hexaploids of the *B. intermedia* and *B. pertusa* complexes. The chromosome numbers of all accessions used in this study were previously determined (Celarier, 1957; Celarier and Harlan, 1955; Celarier, Mehra, and Wulf, in press, and unpublished).

Pollen grains and stomata guard cells were studied from both fresh material and herbarium specimens and the results are given in tables 1 and 2. Although most specimens were only three or four years old, it seems likely that, under proper storage conditions, only a negligible amount of change would be expected with the age of the specimen.

D. annulatum complex

In the present report three diploid, eight tetraploid, and two hexaploid accessions were studied. The data are presented in table 1, and figures 1–8 show their general appearance.

Pollen grain size was quite variable in all accessions with a range of approximately 10μ. However the means were similar in all accessions of one ploidy level, and quite different between polyploids (figs. 1–3). The diploids means ranged from 32.0 to 33.0μ, the tetraploids from 36.2 to 39.9μ, and the hexaploids from 42.9 to 48.7μ. Pollen grains from herbarium specimens were almost always smaller than fresh material but usually the mean values were of less than one micron difference.

There was also variation in stomata guard cell size but it was much less than in pollen grains and was in general, less than five microns. Again the mean values were quite distinct at the different ploidy levels. In the fresh material the means in the diploids varied from 23.7 to 24.8μ, in the tetraploids from 30.0 to 32.0μ, and the hexaploids varied from 36.8 to 45.9μ. The same kind of variation was seen in the herbarium specimens but guard cells were in all cases considerably s maller than in fresh material (figs. 4–6).

TABLE 1. Comparison of pollen grains and guard cells with degree of ploidy in *Dichanthium annulatum*.

A-No.	Location	2n	Pollen Grain Size—μ				Guard Cell Size—μ			
			Fresh		Specimens		Fresh		Specimens	
			range	mean	range	mean	range	mean	range	mean
3242	Calcutta, India	20	26.5–37.1	32.4	26.5–37.1	32.2	22.8–25.2	24.3	10.8–16.8	13.6
3965b	Calcutta, India	20	29.1–37.1	32.9	29.1–37.1	32.8	22.8–26.4	24.8	13.2–15.6	14.0
5396	Belatal, India	20	26.5–37.1	33.0	26.5–37.1	33.2	21.6–25.2	23.7	12.0–16.8	14.6
5437	Lucknow, India	40	31.8–47.7	39.0	31.8–42.4	38.6	30.0–32.4	31.0	18.0–22.8	20.6
5797	Bombay, India	40	31.8–42.4	36.3	31.8–42.4	36.3	28.8–33.6	31.8	22.8–26.4	24.1
4600	Lucknow, India	40	31.8–42.4	36.2	31.8–42.4	36.3	27.6–32.4	30.0	19.2–24.0	20.9
3789	Giza, Egypt	40	31.8–42.4	37.9	31.8–42.4	37.2	28.8–33.6	30.7	18.0–24.0	21.0
4082	South Texas (Int.)	40	37.3–42.4	39.3	31.8–42.4	38.6	30.0–34.8	31.4	18.0–22.8	20.3
4099	Punjab, India	40	37.1–45.0	39.9	37.1–45.0	39.4	28.8–36.0	32.0	18.0–20.4	19.6
3182	N. Gallilea, Israel	40	31.8–42.4	37.1	31.8–42.4	36.4	30.0–33.6	31.7	20.4–27.6	22.4
5295	Coimbatore, India	40	31.8–42.4	37.1	31.8–42.4	36.4	28.8–32.4	30.4	16.8–20.4	18.8
3716	Southern Rhodesia	60	31.8–53.0	48.7	37.1–47.7	45.1	34.8–38.4	36.8	26.4–34.8	29.6
4080	South Africa	60	37.1–53.0	42.9	39.7–47.7	43.8	43.2–48.0	45.9	24.0–30.0	26.2

TABLE 2. Comparison of pollen grains and guard cells with degree of ploidy in *Bothriochloa* species.

A-No.	Location	2n	Pollen Grain Size—μ				Guard Cell Size—μ			
			Fresh		Specimens		Fresh		Specimens	
			range	mean	range	mean	range	mean	range	mean
	Bothriochloa ischaemum									
561	Mus, Turkey	40	31.8–42.4	37.1	31.8–39.7	35.4	24.0–26.4	25.0	15.6–20.4	19.0
5704	Peking, China	40	31.8–42.4	36.7	34.4–42.4	36.6	27.6–30.0	28.7	15.6–20.4	18.0
726	Amoy, China	50	34.4–47.7	38.2	31.8–45.0	39.3	28.8–31.2	30.2	19.2–22.8	21.3
6459	Hong Kong, China	50	31.8–45.0	37.8	31.8–39.7	35.5	27.6–32.4	29.0	18.0–21.6	20.6
2582	Formosa	60	37.1–47.7	43.9	37.1–47.7	43.9	28.8–32.4	30.7	19.2–24.0	21.8
1347	Triangle City, China	60	37.1–47.7	41.9	37.1–45.0	40.9	31.2–36.0	31.8	18.0–24.0	22.0
	Bothriochloa intermedia									
5409	Bareilly, India	40	31.8–39.7	34.7	31.8–37.1	34.4	24.0–27.6	25.8	14.4–16.8	15.2
5450	Delhi, India	40	31.8–42.4	36.9	31.8–39.7	35.0	22.8–28.8	25.5	13.2–15.6	14.7
4596	Gatton, Australia	60	34.4–47.7	42.0	34.4–47.7	41.9	25.2–31.2	29.1	18.0–21.6	19.6
4597	Gatton, Australia	60	31.8–47.7	39.7	31.8–42.4	39.7	30.0–36.0	32.8	18.0–21.6	20.1
	Bothriochloa pertusa									
4806	Hyderabad, India	40	31.8–39.7	35.8	31.8–37.1	35.0	24.0–27.6	25.8	12.0–15.6	13.9
3185	Cuba (Int.)	40	34.4–42.4	37.6	31.8–42.4	36.5	21.6–27.6	24.3	13 2–15.6	14.2
4905	South Africa	60	31.8–45.0	39.4	31.8–42.4	37.1	28.8–32.4	30.0	18.0–21.6	20.2
3704	South Africa	60	39.7–47.7	44.3	37.1–47.7	42.2	34.8–38.4	34.9	20.4–22.8	21 6

Although the diploids, tetraploids, and hexaploids could be easily distinguished from one another in the herbarium materials it is obvious that comparisons between herbarium and fresh materials cannot be made until a correction factor is established.

B. ischaemum complex

Two accessions each were studied in the tetraploids, pentaploids and hexaploids (table 2). As in *D. annulatum* there is considerable variation in pollen grain size (ca. 10–15μ) whereas the range in variation in the guard cells is small (less than 5μ). The means however were rather constant for both, but different in different ploidy levels.

In the tetraploids the means of pollen grain size ranged from 36.7 to 37.1μ for fresh materials and 35.4 to 36.6μ for specimens. The pentaploids ranged from 37.8 to 38.2μ for fresh materials and 35.5 to 39.3μ for specimens, and in the hexaploids the range was 41.9 to 43.9μ for fresh material and 40.9 to 43.9μ from specimens.

Stomata guard cells were also distinct but as in *D. annulatum* showed a big difference from fresh material to specimens.

In the tetraploids the means ranged from 25.0 to 28.7μ in fresh materials and 18.0 to 19.0μ in specimens. The pentaploids ranged from 29.0 to 30.2μ in fresh materials and 20.6 to 21.3μ in specimens, and the hexaploids ranged from 30.7 to 31.8μ in fresh material and 21.8 to 22.0μ in specimens.

In *B. ischaemum* it seems that pentaploids cannot be easily separated from hexaploids on guard cell size alone but by the use of both pollen grain and stomata guard cells the separation is fairly reliable.

B. intermedia complex

In this species only two tetraploids and two hexaploids were used. The tetraploids were readily distinguishable from the hexaploids with both pollen grain size and stomata guard cell size. Variation in both was rather similar to that seen in *D. annulatum* and *B. ischaemum*.

The tetraploids had a range in mean pollen grain size of 34.7 to 36.9μ in fresh materials and 34.4 to 35.0μ in specimens; whereas the hexaploids ranged from 39.7 to 42.0μ in fresh material and 39.7 to 41.9μ in specimens.

The range of the means in guard cell size were 25.5 to 25.8μ in fresh materials and 14.7 to 15.2μ in specimens for the tetraploids and for the hexaploids were from 29.1 to 32.8μ for fresh materials and 19.6 to 20.1μ for specimens.

B. pertusa complex

In this species complex two tetraploids and two hexaploids were used and the results were similar to those found in the other species.

The range in pollen grain means was 35.8 to 37.6μ for fresh material

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Figs. 1–8. Pollen grains and stomata guard cells in *Dichanthium annulatum*. Figs. 1–3. Pollen of the three ploidy levels. X300. Fig. 1 diploid. Fig. 2 tetraploid. Fig. 3 hexaploid. Figs. 4–6. Comparison of stomata guard cells from fresh mounts and plastic peels of herbarium specimens in the three ploidy levels. X1350. Fig. 4 diploid. a. fresh material. b. plastic peel of specimen. Fig. 5 tetraploid. a. fresh material. b. plastic peel of specimen. Fig. 6. hexaploid. a. fresh material. b. plastic peel of specimen. Figs. 7–8. Comparison of plastic peel of fresh material (Fig. 7) with plastic peel of specimen (Fig. 8) in the hexaploid. X300.

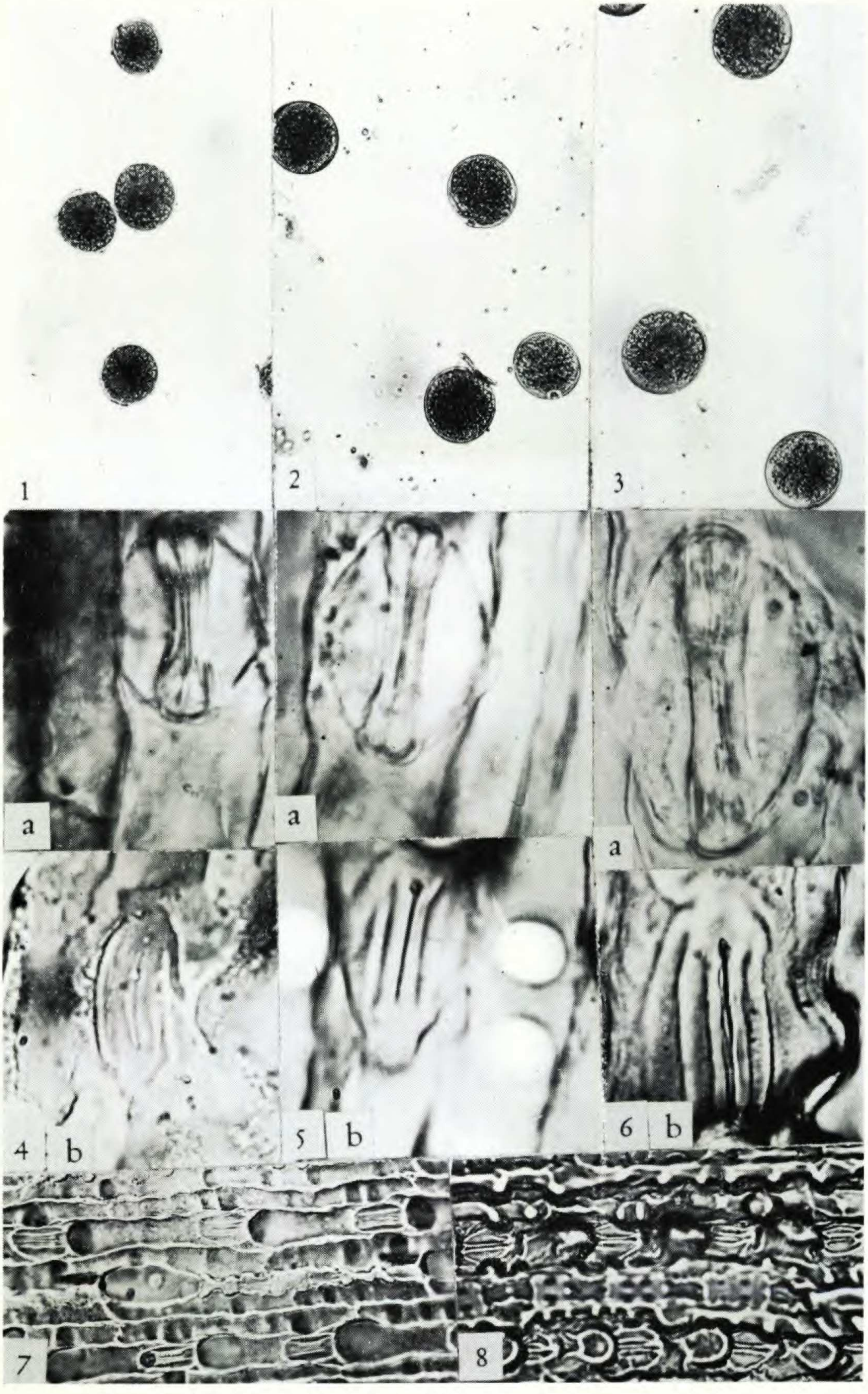

Fig. 1–8. For explanation see opposite page.

and 35.0 to 36.5μ for specimens in the tetraploids, and 39.4 to 44.3μ for fresh material and 37.1 to 42.2μ for specimens in the hexaploids.

The stomata guard cell size was also distinct with means ranging from 24.3 to 25.8μ in fresh material and 13.9 to 14.2μ in specimens for the tetraploids; whereas, the hexaploids ranged from 30.0 to 34.9μ in fresh material and 20.2 to 21.6μ in specimens.

Discussion and Conclusions

It has been shown that both pollen grain and stomata guard cell size are fairly reliable indicators of the degree of ploidy in several of the Old World species of the genera *Bothriochloa* and *Dichanthium*. It has also been shown by Gould (1957) that pollen grain size is useful in determining the degree of ploidy in several of the American species of *Bothriochloa*.

In the species studied there was no difficulty in distinguishing between diploids, tetraploids and hexaploids. However in *B. ischaemum* the differences between the pentaploids and hexaploids were not so distinct, but by the use of both pollen grain and guard cell sizes a fairly reliable conclusion could be drawn.

In general it was possible to place the materials studied in their proper ploidy levels by pollen grain and guard cell size regardless of the species involved. However there were exceptions to this, such as the tetraploid *D. annulatum* A-4099 with pollen grains 39.9μ and guard cells of 32.0μ and the hexaploid *B. intermedia* A-4597 with pollen grains of 39.7μ and guard cells of 32.8μ.

The impression technique for measuring guard cells is shown to be quite reliable but the actual measurements were in all cases much less than those made from fresh material. In order to determine what portion of this decrease in length was due to the technique and what portion was due to the drying of the specimens, measurements were also made from plastic strips taken from fresh material.

In the diploid *D. annulatum* A-3242 the mean guard cell measurements from fresh material was 24.3μ whereas the plastic strip measurements from specimens was 13.6μ. Plastic strip measurements of fresh material of this accession were found to be 20.4μ. From this it is seen that a considerable portion of this decrease in size is due to the technique itself but that most of it is probably due to the shrinkage in drying of the specimens. A similar condition was found in the hexaploid (figs. 7–8) but only a few measurements were made.

These studies seem to warrant certain recommendations in the procedures used in studies where it is desirable to determine chromosome numbers from herbarium specimens. The following appear to be significant:

1. Pollen grain and stomata guard cell size are usually reliable indicators of polyploidy, and the use of both would be expected to give much more dependable results than either alone.

2. Actual chromosome counts should be made from at least a few plants of several polyploid levels. Pollen grain and guard cell measurements from these plants can serve as a standard.

3. Data should be calculated in terms of ranges and means. This seems to be especially important in studies of pollen grains.

4. Guard cells from herbarium specimens can be reliably measured by the impression technique but when compared with fresh material a correction factor must be taken into account to offset the shrinkage.

5. Conclusions regarding chromosome number based on cell size should be transferred to a second species with extreme caution, unless some chromosome counts of the second species have been made so that a standard can be established.

6. Data concerning cell size would be a valuable addition to a monograph even if the chromosome numbers of the taxa involved are not known, in that they may offer a suggestion of polyploidy and will be available if cytological studies are made in the future.

Summary

Data are presented that demonstrate a correlation between the degree of polyploidy and size of pollen grains and stomata guard cells in four species complexes of the grass genera *Dichanthium* and *Bothriochloa*. These studies were made both from living material and dried herbarium specimens.

An impression technique using plastic strips is outlined for the study of stomata guard cells from herbarium specimens. This technique gives reliable measurements without damage to the specimens.

Some of the limitations to the use of cell size as a gauge of polyploidy are discussed and certain recommendations are offered based on present studies.—DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, AGRIC. EXP. STA., OKLAHOMA STATE UNIVERSITY, STILLWATER.